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Research Article

RESEARCH ARTICLE

Trafficking of *Estrella lausannensis* in human macrophages

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One sentence summary: *Estrella lausannensis* is efficiently replicating in human macrophages, by escaping the endocytic pathway, but induces a rapid cytopathic effect, likely by pyroptosis.
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ABSTRACT

Estrella lausannensis is a new member of the *Chlamydiales* order. Like other *Chlamydia*-related bacteria, it is able to replicate in amoebae and in fish cell lines. A preliminary study investigating the pathogenic potential of *Chlamydia*-related bacteria found a correlation between antibody response to *E. lausannensis* and pneumonia in children. To further investigate the pathogenic potential of *E. lausannensis,* we determined its ability to grow in human macrophages and its intracellular trafficking. The replication in macrophages resulted in viable *E. lausannensis*; however, it caused a significant cytopathic effect. The intracellular trafficking of *E. lausannensis* was analyzed by determining the interaction of the *Estrella*-containing inclusions with various endocytic markers as well as host organelles. The *E. lausannensis* inclusion escaped the endocytic pathway rapidly avoiding maturation into phagolysosomes by preventing both EEA-1 and LAMP-1 accumulation. Compared to *Waddlia chondrophila,* another *Chlamydia*-related bacteria, the recruitment of mitochondria and endoplasmic reticulum was minimal for *E. lausannensis* inclusions. *Estrella lausannensis* appears to use a distinct source of nutrients and energy compared to other members of the *Chlamydiales* order. In conclusion, we hypothesize that *E. lausannensis* has a restricted growth in human macrophages, due to its reduced capacity to control programmed cell death.

Keywords: *Chlamydia*-related bacteria; intracellular bacteria; mitochondria; Golgi; cytopathic effect

INTRODUCTION

Estrella lausannensis is a new member of the *Chlamydiales* order (Lienard *et al.* 2011b), isolated from a Spanish river sample by amoebal coculture (Corsaro *et al.* 2009). With *Criblamydia sequanensis* that was also recovered by amoebal co-culture from the Seine river in Paris (Thomas, Casson and Greub 2006), *E. lausannensis* is one of the two species assigned to the *Criblamydiaceae* family (Thomas, Casson and Greub 2006; Lienard *et al.* 2011b). The number of members in the *Chlamydiales* order has been constantly growing in recent years (Everett, Bush and Andersen 1999; Greub 2010). Environmental samples and metagenomics projects have provided a tremendous source of new members and families within the *Chlamydiales* order, showing the high biodiversity and widespread occurrence of this clade of strict intracellular bacteria (Lienard *et al.* 2011b; Pizzetti *et al.* 2012; Lagkouvardos *et al.* 2013). However, the majority of the studies only offered information at the phylogenetic level without isolation and functional studies. Initial studies with a few members of other families besides the *Chlamydiaceae* have already shown that the replication strategies of these obligate intracellular bacteria can be diverse and may reflect their adaptation to specific hosts (Kebbi-Beghdadi, Bertelli and Greub 2012).

Criblamydiaceae exhibit the same replication cycle with infectious elementary bodies (EBs) and replicative reticulate bodies (RBs) as other members of the *Chlamydiales* order (Lienard *et al.* 2011b). *Chlamydiales* reside throughout the infection within

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a vacuole called inclusion. The nature of the inclusion and the organelles that interact with it varies between members of the *Chlamydiales* order (Kebbi-Beghdadi, Bertelli and Greub 2012). For example, *Chlamydia trachomatis* inclusions associate with Golgi fragments (Hackstadt, Scidmore and Rockey 1995), *Waddlia chondrophila* with the endoplasmic reticulum (ER; Croxatto and Greub 2010), whereas *Parachlamydia acanthamoebae* remains in the endocytic pathway, but prevents the acidification of the inclusion and the acquisition of lysosomal hydrolases, such as cathepsin (Greub *et al.* 2005). For many other members of the *Chlamydiales* order, the trafficking in the host cell remains unknown.

Upon an initial report on the growth of *E. lausannensis* in several amoebal hosts (Lienard *et al.* 2011b), Kebbi-Beghdadi, Batista and Greub (2011) showed that *E. lausannensis* also replicates within fish cell lines, and in preliminary studies in endometrial cells and pneumocytes. The wide range of cell types that could be infected by *E. lausannensis* suggests a wider host range than *Chlamydiaceae*. Interestingly, it was shown that its growth rate within amoebae is increasing at higher temperatures (Lienard *et al.* 2011b). Moreover, the presence of *E. lausannensis* or a related *Criblamydiaceae* in human samples was shown in a study on nasopharyngeal swabs from children with pneumonia (Lienard *et al.* 2011a). These observations suggested that *E. lausannensis* could also replicate within phagocytic cells, such as human macrophages and could represent an emerging pathogen. Recently, the genome of *E. lausannensis* became publicly accessible and will provide an important source of information to investigate virulence factors (Bertelli *et al.* 2015).

We therefore decided to investigate the replication of *E. lausannensis* in human macrophages, since they are the first response innate immune cells in the lung. We could demonstrate that macrophages are permissive to *E. lausannensis* and therefore further investigated its intracellular trafficking.

MATERIAL AND METHODS

Cell differentiation and bacterial strains

The human cell line PLB-985 was kindly provided by M. Grez (Frankfurt, Germany). Cells were cultured in RPMI (Gibco-BRL Life Technologies, Basel, Switzerland), 10% FCS (Biochrom, Berlin, Germany) at 37°C with 5% CO_2. To differentiate monocytes into macrophages, cells were exposed to 50 nM PMA for 48 h. The *Chlamydia*-related bacteria *E. lausannensis* (CRIB30) and *W. chondrophila* (ATCC VR-1470) were cultivated in *Acanthamoeba castellanii* (ATCC30010) as previously described (Bertelli *et al.* 2010).

DNA extraction and qPCR

Genomic DNA of *W. chondrophila* and *E. lausannensis* was extracted using the tissue extraction protocol from Wizard SV Genomic DNA kit (Promega, MA, USA). Samples were quantified by qPCR as published before (Goy *et al.* 2009; Lienard *et al.* 2011a).

Infectivity

Macrophages infected with *E. lausannensis* were collected at 0 and 24 h p.i. with the supernatant. The samples were mixed with glass beads to disrupt the cells and filtered on 5 μm filters to remove debris and serially diluted. The serial dilutions were used to infect Vero cells by spinoculation. The infected cells were fixed after 24 h and inclusions containing *E. lausannensis* were stained with in-house polyclonal mouse anti-*Estrella* antibodies and secondary anti-mouse antibodies. All inclusions per coverslip were quantified in duplicates in three different experiments.

Cell death quantification

Propidium iodide (5 μg ml^{-1}) was added to infected or mock-treated cells in a 96 well with RMPI 10% FCS without phenol red. The fluorescence was measured with a FLOUstar Omega microplate reader (BMG Labtech, Offenburg, Germany). Lactate dehydrogenase (LDH) release was measured with the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) according to manufacturer's instructions. Changes in PI or LDH release were tested with an unpaired non-parametric Mann-Whitney test compared to mock-infected cells using GraphPad Prism version 6.0 for Windows.

Antibody staining

Coverslips were blocked in blocking solution (0.3% Triton X-100 and 5% FCS in PBS) for 1 h at room temperature. For early endosomes (EEA1), late endosomes (LAMP-1), Golgi (RCAS1) and ER (PDI) staining, the organelle localization kit (Cell Signaling Technology Inc., Beverly, USA) was used according to manufacturer's instructions with overnight incubation at 4°C with a primary antibody. After organelle staining, coverslips were washed three times in PBS and incubated with in-house mouse polyclonal antibodies against *W. chondrophila* (1:1000) or *E. lausannensis* (1:1000) for 1 h at room temperature. Prior to incubation with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Paisley, Scotland) and secondary antibodies Alexa Fluor 488 goat anti-mouse (1:1000) and Alexa Fluor 594 donkey anti-rabbit (1:1000) (Molecular Probes Leiden, Netherlands) for 1 h at room temperature, coverslips were washed three times in PBS. Finally, coverslips were washed three times in PBS and mounted with MOEWIOL (Sigma-Aldrich, Buchs, Switzerland) on glass slides.

For tubulin (Abcam Inc., Cambridge, UK) staining, the same procedure was used except that the primary antibody (1:200) was incubated with the polyclonal anti-*Waddlia* or anti-*Estrella* for 1 h at room temperature. Actin filaments were directly stained with Phalloidin CF594 (Biotium, Hayward, USA) together with the polyclonal anti-*Waddlia* or anti-*Estrella* antibody at room temperature for 1 h. All subsequent steps with secondary antibodies were performed as described above. For mitochondrial staining, cells were incubated with 250 nM of MitoTracker Red CMXRos (Life Technologies) 30 min prior to fixation with acetone. After 1 h blocking in triton-free blocking solution, coverslips were incubated as described above for bacterial primary and secondary antibodies with anti-*Waddlia* or anti-*Estrella* antibodies.

FM4-64 staining

FM4-64 staining protocols were adapted from Vieria *et al.* (2003) for staining of infected cells. The cells were first infected and then exposed to 5 μg FM4-64 (Life Technologies) for different incubation times to determine the fusion of inclusions with nascent phagosomes. Cells were fixed with 4% PFA and extracellular bacteria were marked with anti-*Estrella* for 1 h. Internal bacteria were marked with Hoechst without permeabilization (Sigma-Aldrich). For detection of fusion with late endosomes or lysosomes, the cells were incubated with FM4–64 for 1 h and then pulsed for 3 h prior to infection. Cells were fixed and marked as described beforehand.

Infection and fixation

Differentiated macrophages grown on glass coverslips were infected with *E. lausannensis* or *W. chondrophila* by spinoculation. Cells were centrifuged for 15 min at 1790 g and further incubated 15 min at 37°C, 5% CO_2, in order to allow bacterial internalization. Cells were washed to eliminate all non-internalized bacteria and fresh medium was added. At different points in time post infection (p.i.), cells were fixed with protocols adapted to each antibody staining. For actin and tubulin staining, cells were fixed in −20°C methanol for 10 min. For MitoTracker Red CMXRos, cells were incubated during 10 min in −20°C acetone. For LAMP-1 (Cell Signaling Technology Inc.) staining, cells were fixed in a 1:1 acetone/methanol solution for 10 min at −20°C. For all other organelle markers, cells were fixed with 4% paraformaldehyde at room temperature for 15 min. For protein disulfide isomerase (PDI) staining, an additional methanol permeabilization (10 min, −20°C) was performed. All fixed samples were washed three times with PBS.

Western blot

A T25 flask of confluent PLB-985 cells differentiated in macrophages was infected with *E. lausannensis* either live or formol inactivated with the previously described method. At different points in time, the supernatant was removed and the cells were lysed in a 500 μl RIPA buffer (Sigma-Aldrich). Proteins were separated on a 12% pre-cast gel (Biorad Laboratories, Rheinach, Switzerland) and transferred on a nitrocellulose membrane (Membrane Solutions Inc., North Bend, USA). The membrane was incubated over night with 1:1000 RCAS1 antibody (Cell Signaling) and revealed by chemiluminescence with an anti-rabbit-HRP antibody (Biorad). The membrane was stripped prior to the control staining with GAPDH (Sigma-Aldrich). The antibody was incubated overnight at a 1:250 dilution and revealed with anti-mouse-HRP (Biorad) by chemiluminescence. Band intensity was quantified with ImageJ (Rasband 1997–2014). Significance of band intensity changes was assessed using an unpaired non-parametric Mann-Whitney test.

Microtubules and actin disruption

A total of 20 μM of nocodazole (Sigma-Aldrich) and 10 μM cytochalasin D (Sigma-Aldrich) were used to disrupt the cytoskeleton of infected macrophages. The drugs were added at different points in time p.i. and the cells were fixed with methanol 12 h p.i. The fixed coverslips were blocked and the bacteria were stained with mouse anti-*Estrella* antibody and secondary Alexa488 anti-mouse antibody as described above. The mounted coverslips were analyzed with an automated Axiovision (Zeiss, Feldbach, Switzerland). Four images of eight by eight tiles with a 20% overlap were taken with a 400× magnification. Images were acquired in the FITC and DAPI channels. The acquired images were then further processed with ImageJ to split the channels, define the cell outline and the size of the inclusions (area). The inclusions were divided into large, medium and small according to the surface covered by the inclusion and normalized by the number of infected cells. The number of inclusions determined with ImageJ was normalized to 100 infected cells. Significance was tested with an unpaired non-parametric Mann-Whitney test compared to 0.1% DMSO-treated cells. GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis.

Lysosome and ER-Golgi traffic disruption

To disrupt lysosomal acidification, the drug bafilomycin A (100 nM) was added to infected cells or mock-infected cells (0.1% DMSO) for 12 h or 4 h p.i. For the disruption of the ER-Golgi trafficking, the drug brefeldin A (1 μg ml^{-1}) was used for the same period of time. Cells were then fixed with 4% PFA and bacteria were stained with in-house polyclonal anti-*E. lausannensis* antibodies for 1 h at room temperature. Secondary Alexa Fluor 488 goat anti-rabbit and DAPI were added for 1 h at room temperature. Coverslips mounted in MOEWIOL were imaged with a scanning Axiovision microscope. The tiled images were analyzed with ImageJ to determine the amount of cells present (DAPI staining) and the number of inclusions. Inclusions were separated according to size (area) and normalized according to the number of cells present on the slide.

Confocal microscopy and colocalization analysis

Images were acquired with a confocal microscope Zeiss LSM510 Meta or Zeiss LSM710 and analyzed with Zen 2009 Light edition software. For Golgi fragmentation, cells were analyzed with an epifluorescent microscope (Leitz Diaplan, Leica, Switzerland). Colocalization of all bacteria containing vacuoles and organelles was assessed on 100 infected cells in three independent experiments. Statistical analyses of the acquired data were performed with GraphPad Prism version 6.0.

RESULTS

Estrella lausannensis replicates within macrophages

PLB-985 monocytes were differentiated into macrophage-like adherent cells by adding 50 nM PMA for 48 h. The macrophages were infected with *E. lausannensis* with a bacterial load of two to three bacteria per cell and an infection rate above 50%. *Estrella lausannensis* readily differentiated into RBs 4 h p.i. The bacteria started to replicate shortly after, and at 12 h p.i. large inclusions were visible (Fig. 1a). At 16 h p.i., some of the bacteria were already redifferentiating into EBs. The growth was more than 1 log (24-fold increase) in 24 h (Fig. 1b). *Waddlia chondrophila*, another member of the *Chlamydiales* order, previously shown to multiply in macrophages (Goy, Croxatto and Greub 2008), was used as a control and replicated as well in this cell line (Fig. 1a). Within the same time frame, *W. chondrophila* replication was about 1 log higher than *E. lausannensis* at the same infection rate and bacterial load (Fig. 1b). To determine the infectivity of the newly produced *E. lausannensis*, the infected cells were collected at 24 h and used to re-infect Vero cells. We observed 20 times more inclusion forming units (IFU) with *E. lausannensis* derived from infected macrophages collected at 24 h p.i. ($12 \pm 5.3\ 10^4$ IFU) compared to those collected at 0 h p.i. ($6 \pm 3.2\ 10^3$ IFU).

At 4 and 6 h p.i., a significant fraction of *E. lausannensis* infected cells showed a fragmented nucleus. This was not observed in non-infected cells or in cells infected with *W. chondrophila* (Fig. 2a and b). To confirm the cytopathic effect of *E. lausannensis*, we determined the amount of propidium iodide (PI) that was taken up by infected cells (Fig. 2c). The proportion of PI positive cells was significantly higher in *E. lausannensis* infected macrophages compared to cells infected with *W. chondrophila* or formol-inactivated *E. lausannensis*. These data were further confirmed by measuring LDH release, another cytopathic marker. Only cells infected with live *E. lausannensis* caused a significant

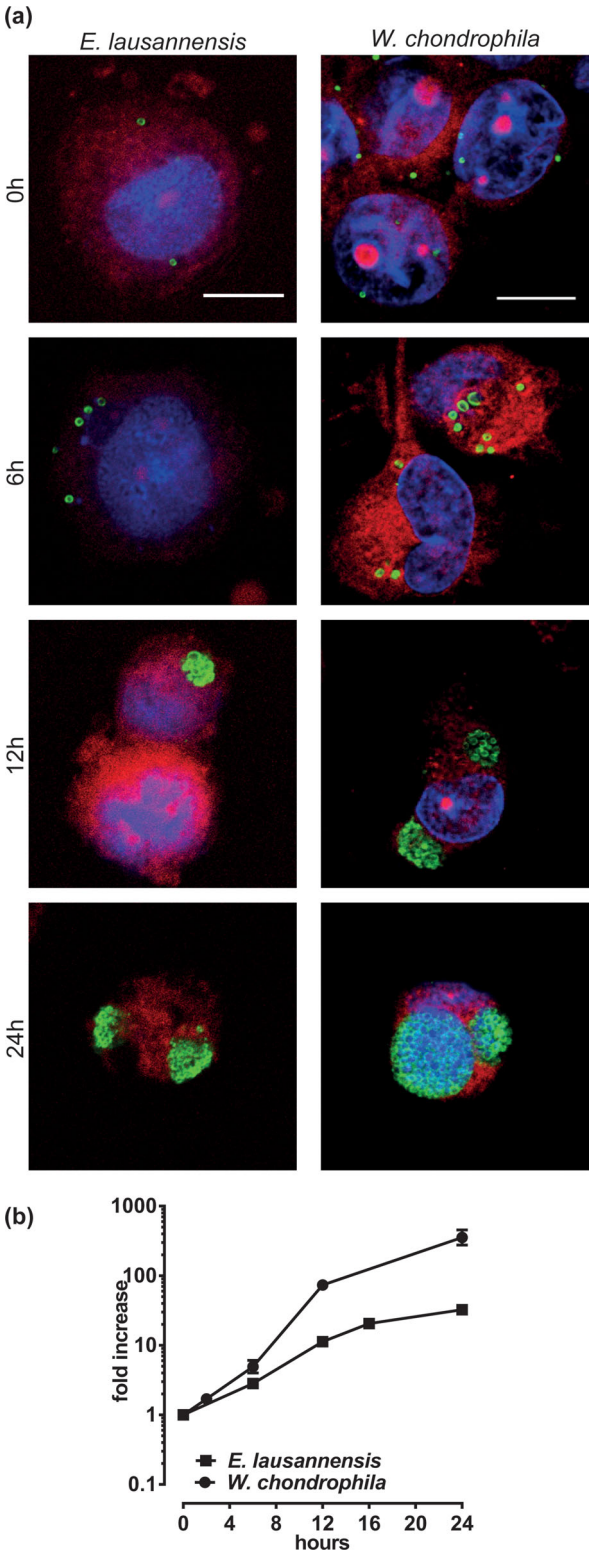

Figure 1. Growth of *E. lausannensis* in human macrophages. (a) Growth of *E. lausannensis* and *W. chondrophila* occurs in the same time frame. Bacteria differentiate into RBs about 6 h p.i. Replication initiates rapidly with large inclusions already visible at 12 h p.i. At 24 h p.i., most inclusions are mature, containing infectious EBs. Bacteria are stained with in-house polyclonal rabbit or mouse antibodies (green). Cells are marked with Texas Red conjugated-concanavalin A (red) and DAPI (blue). Scale bar 10 μm. Pictures were taken at 1000× magnification. (b) Bacterial growth was confirmed by specific qPCR. Data are the means +/− SEM of at least three independent experiments performed in duplicates.

release of LDH (Fig. 2d). Infections with *W. chondrophila* or with formol-inactivated bacteria did not cause cell death.

Estrella lausannensis escapes endosomal compartments

Estrella lausannensis-containing inclusions did not accumulate the early endosomal marker EEA1 as seen for *W. chondrophila*-containing vacuoles (Fig. 3a). The percentage of EEA1 positive *E. lausannensis* bacteria-containing vacuoles (BCV) rose from 5 to 11% in the first 30 min p.i. and subsequently dropped again at 1 h p.i. Conversely, as previously described (Croxatto and Greub 2010), up to 30% of *W. chondrophila* BCV showed an EEA1 staining 15 min p.i., but it was then rapidly lost. We could not analyze the trafficking of inactivated *E. lausannensis,* since these bacteria were rapidly recycled back outside the macrophages or degraded.

Even though the *E. lausannensis* BCV did not strongly accumulate EEA1, we investigated if it still might acquire lysosomal markers later in the infection. We did not observe any increase in LAMP1 staining up to 6 h p.i., when the bacteria were already differentiated into RBs. Only about 10% of *E. lausannensis* BCVs showed a LAMP1 staining and the percentage remained stable throughout time (Fig. 3b). To determine if the *E. lausannensis* BCV was completely dissociated from the endosomal network, we used the solvochromic FM4-64 compound in live cells. If *E. lausannensis* BCV can still fuse with endosomal compartments, it will acquire FM4-64 that enters the cells through the endosomal pathway upon integration into the plasma membrane (Vieira *et al.* 2003). Since FM4-64 can also integrate into the bacterial membrane, staining was performed without permeabilization in order to distinguish between the extra- and the intracellular bacteria. We observed a strong accumulation of FM4-64 in the BCV. The majority of inclusions displayed a FM4-64 positive staining for up to 1 h p.i. (Fig. 3c). Even though the EEA1 staining started to decrease already after 30 min, *E. lausannensis* BCV was still interacting with the endosomal network for a longer period, since the FM4-64 staining was still strong at 1 h p.i. To determine if *E. lausannensis* BCV could fuse with preexisting lysosomes instead of maturating into a lysosome, we performed a pulse and chase with FM4-64. The cells were incubated with FM4-64 for 1 h and then chased for 3 h prior to infection. About half of the inclusions that were fixed shortly after infection were FM4-64 positive. The amount dropped rapidly over the next hour (Fig. 3d).

Estrella lausannensis infected cells exhibit Golgi fragmentation

Once we determined that *E. lausannensis* escapes the endosomal route early on, we analyzed the interaction of the BCV with several host organelles to define the nutriment source of this obligate intracellular bacteria. For *E. lausannensis*, we observed a significant Golgi fragmentation at 4 h p.i. (Fig. 4a). However, the fragmented Golgi did not accumulate around the BCV, contrarily to what is known for *Chlamydiaceae* (Ying *et al.* 2008). Interestingly, we also observed a Golgi degradation in macrophages infected with *W. chondrophila*, although not to the same extent (Fig. 4a). Here as well the BCV did not accumulate Golgi fragments. Further the fate of RCAS1, a golgic protein, was followed by Western blot (Fig. S1a, Supporting Information). There was a degradation observable in *E. lausannensis* infected cells, but it was not significant compared to mock-infected cells (Fig. S1b, Supporting Information).

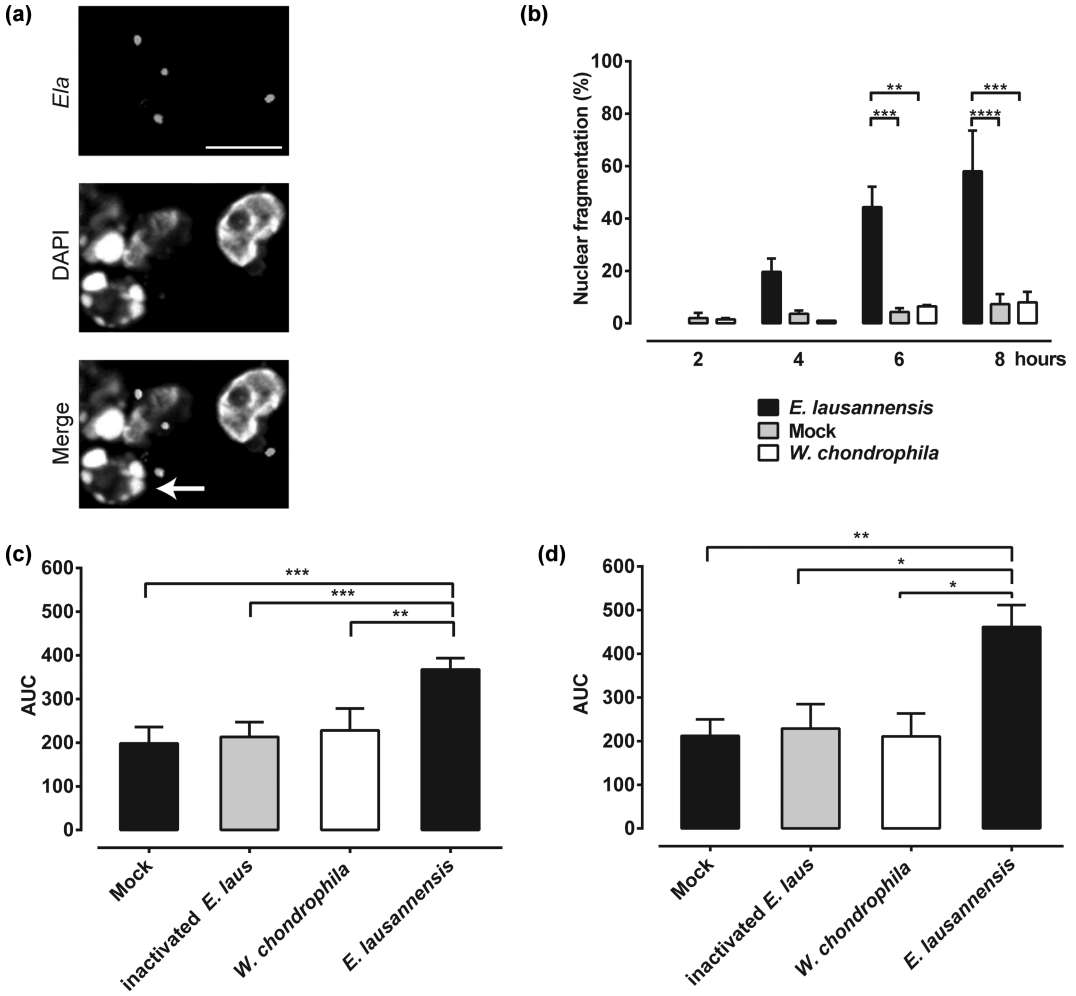

Figure 2. Cytopathic effect of *E. lausannensis* (*E. laus.*). (**a**) Infected cells showed fragmented (white arrow) or condensed nuclei 8 h p.i., prior to bacterial replication. Scale bar 10 μm. Pictures were taken at 1000× magnification. (**b**) *Estrella lausannensis* causes a significant increase of cells with fragmented nuclei at 6 h p.i. compared to *W. chondrophila* and uninfected cells. The percentage of infected cells with fragmented nuclei was quantified in three independent experiments for 100 infected cells each with SEM. (**c**) Quantification of the cytopathic effect was performed with the impermeable nucleic intercalating agent PI. *Estrella lausannensis* infected cells displayed a significant accumulation of PI early p.i. compared to cells non-infected (mock) or infected with inactivated *E. lausannensis* in the first 16 hours. The total effect was quantified by measuring the area under the curve (AUC). The data are the means +/− SEM of three independent experiments performed in triplicates. (**d**) Cytopathic effect was further confirmed by monitoring LDH release. Data are the means +/− SEM of three independent experiments performed in triplicates. The cytopathic effect was absent in cells infected with *W. chondrophila* or infected with formol-inactivated *E. lausannensis*. Significance was tested with an unpaired non-parametric Mann-Whitney test compared to mock-infected cells (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Estrella lausannensis is less associated with the ER and mitochondria than *W. chondrophila*

We investigated the association of *E. lausannensis* BCV with the ER, which is an important source of membranes and proteins, by determining the colocalization of the inclusion with PDI, a marker of the ER. For *E. lausannensis* BCV, we observed less than 20% of colocalization, compared to up to 60% for *W. chondrophila* inclusions at 8 h p.i. (Fig. 4b). We also determined the colocalization of the *E. lausannensis* inclusions with mitochondria that represent a second source of energy and lipids. The recruitment of mitochondria was much less pronounced in *E. lausannensis* and occurred at later stages (Fig. 4c) than what is observed with *W. chondrophila*. The recruitment is observed mainly at 6 h post *E. lausannensis* infection, whereas *W. chondrophila* (used as a positive control) already recruits mitochondria 2 h p.i.

Disruption of cytoskeleton components delays *E. lausannensis* replication

The interaction of the BCV with the different cellular organelles and compartments is regulated by the cytoskeleton. The cytoskeleton either transports the inclusion to a given location or transports the organelles to the inclusion. For the *Chlamydiales*, several components of the cytoskeleton have been shown to be relevant. *Chlamydia trachomatis* use microtubuli to reach the microtubule organization center (MTOC) (Grieshaber, Grieshaber and Hackstadt 2003). For *W. chondrophila*, a single disruption of microtubuli or actin is not enough to prevent mitochondria recruitment, the recruitment being abolished only when both cytoskeletal elements are perturbed (Croxatto and Greub 2010). Microtubuli can be disrupted by the drug nocodazole and actin filaments by cytochalasin D. We analyzed the

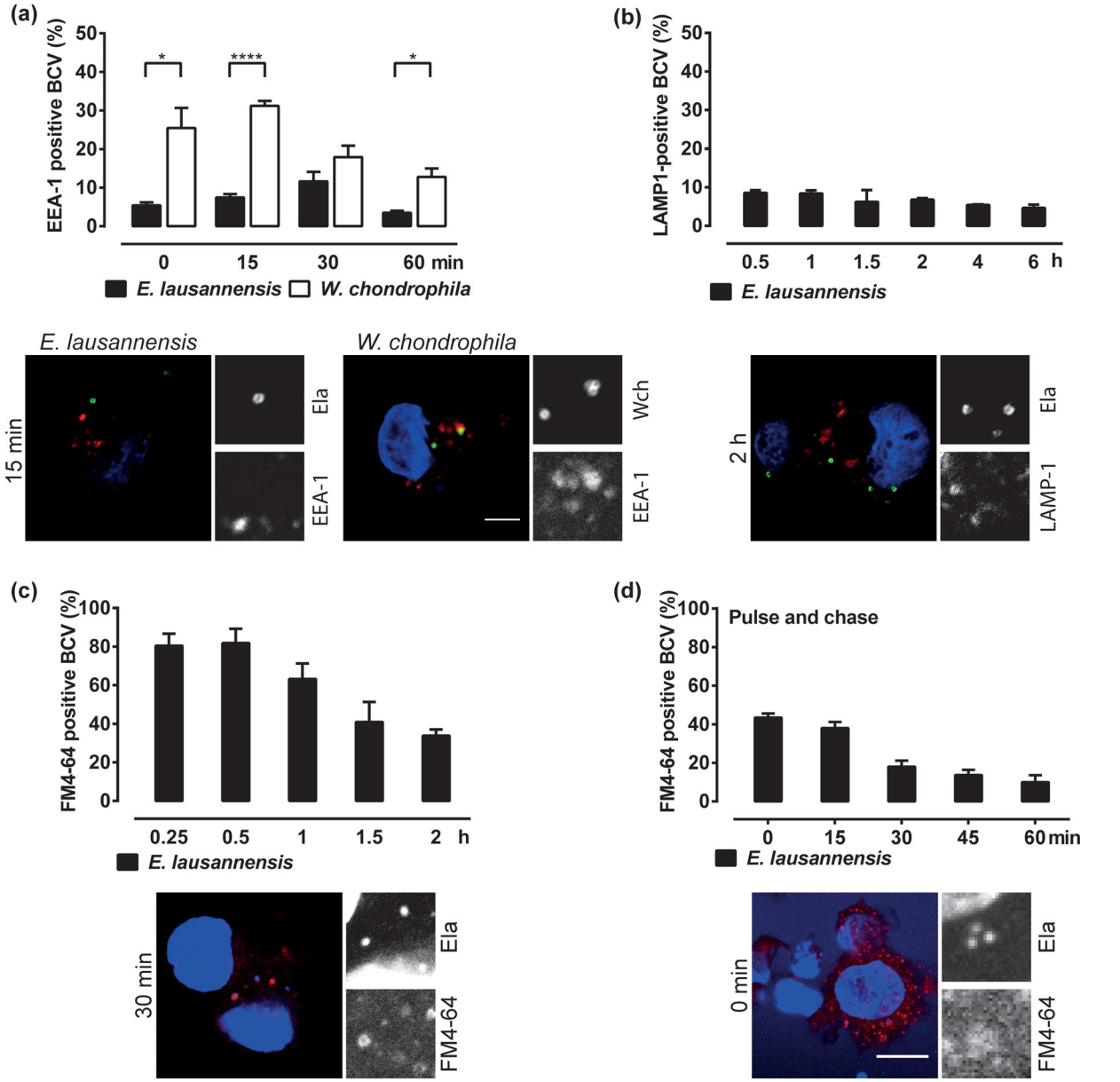

Figure 3. Early intracellular trafficking of *E. lausannensis*. (**a**) Infected cells were fixed at early time points to determine EEA-1 (red) localization on bacterial inclusions (green). *Waddlia chondrophila* (Wch) significantly co-localizes with EEA-1, while *E. lausannensis* (Ela) does not. (**b**) LAMP-1 (red) staining was determined in *E. lausannensis* (green) vacuoles. No accumulation of the lysosomal marker in the BCV was observed up to 6 h p.i (**c**) Acquisition of FM4-64 by *E. lausannensis* inclusions was followed during 2 h p.i. Internalized bacteria (stained in blue with Hoechst) rapidly acquire a strong FM4-64 staining (red). Extracellular bacteria were stained with polyclonal mouse anti-*Estrella* antibody. (**d**) A pulse and chase treatment of macrophages with FM4-64 allowed staining of late endosomes and lysosomes. Cells were subsequently infected with *E. lausannensis* and fusion with previously formed lysosomes and late endosomes was quantified. About half of the internalized *E. lausannensis* fused with these late endocytic compartments. The fusion event was transient as the amount of FM4-64 positive inclusions diminished rapidly. All experiments were performed in triplicates on 100 infected cells. Results are presented with SEM. Images were acquired at a 630× magnification (scale bar 10 μm).

progression of *E. lausannensis* infection in presence of one or both of these drugs by counting, at 12 h p.i., the number of inclusions of defined size (small, middle, large). Small inclusions contain 5–10 replicating bacteria, medium size inclusions encompass 10–20 bacteria, and large inclusions between 20 and 50 bacteria. When cells were treated with nocodazole alone, the number of inclusions was overall not altered (Fig. S2 e–h, Supporting Information). Nevertheless, there were significantly more small inclusions when cells were treated with nocodazole between 0 and 2 h p.i. ($P < 0.041$) (Fig. S2e, Supporting Information) and there were significantly less medium size inclusions upon treatment with nocodazole between 4 and 12 h p.i. ($P < 0.048$) (Fig. S2f, Supporting Information). On the other hand, early treatment with cytochalasin D significantly reduced growth of *E. lausannensis*, even when cells were only exposed to the drug during 2 h (Fig. S2d, Supporting Information). Later exposures to this drug did not affect growth of *E. lausannensis*. Combination of the two drugs blocked replication (Fig. 5a–c) when applied early in infection and further blocked progression of replication when applied at later time points, since significantly less large inclusions were observed (Fig. 5c). Interestingly, when both filaments were disrupted, the growth of

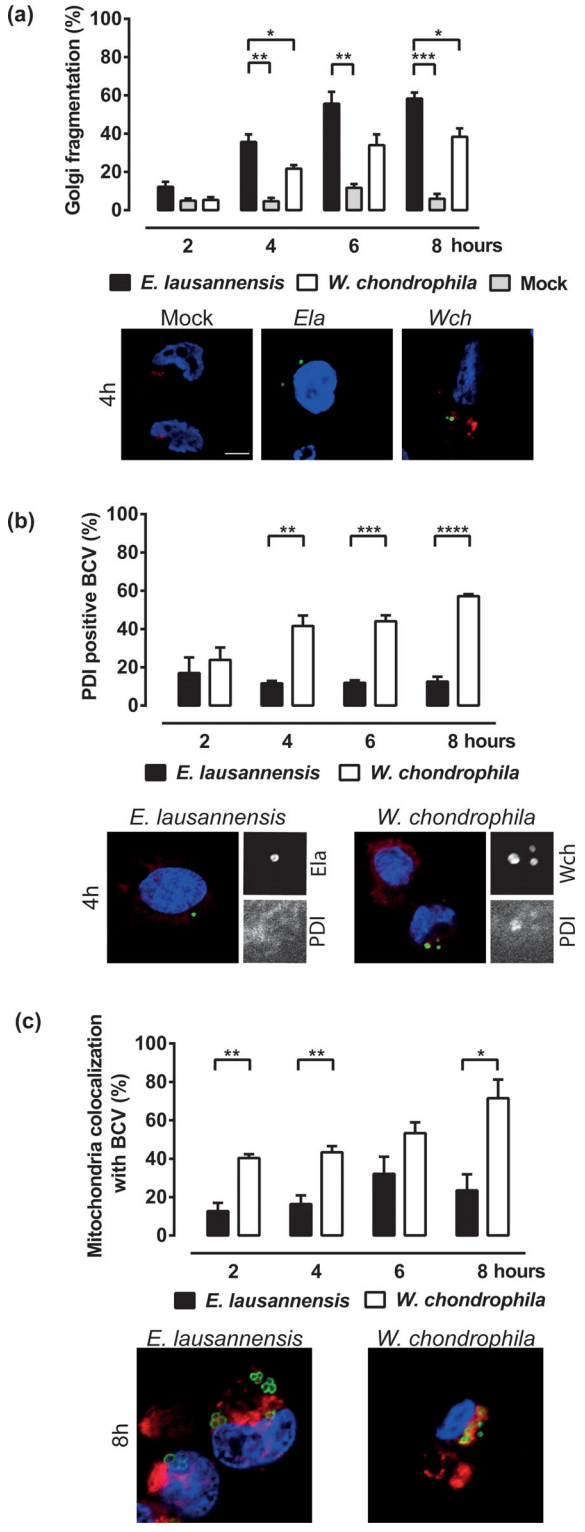

Figure 4. Interaction of *E. lausannensis* inclusion with host organelles. (a) Infection with *E. lausannensis* (green, Ela) causes Golgi (red) fragmentation without recruitment of the Golgi fragments to the inclusion. Partial Golgi fragmentation is also observed in macrophages infected with *W. chondrophila*. (b) Co-localization of ER marker PDI (red) with BCV was not observed for *E. lausannensis* (green) but reached 60% for *W. chondrophila* at 8 h p.i. (c) Mitochondria (MitoTracker Red) are recruited to a lesser extent by *E. lausannensis* inclusions (green) compared to *W. chondrophila*. Nuclei are marked with DAPI (blue). All experiments were performed in triplicates on 100 infected cells. Results are presented with SEM. Images were acquired at a 630× magnification. (scale bar 10 μm).

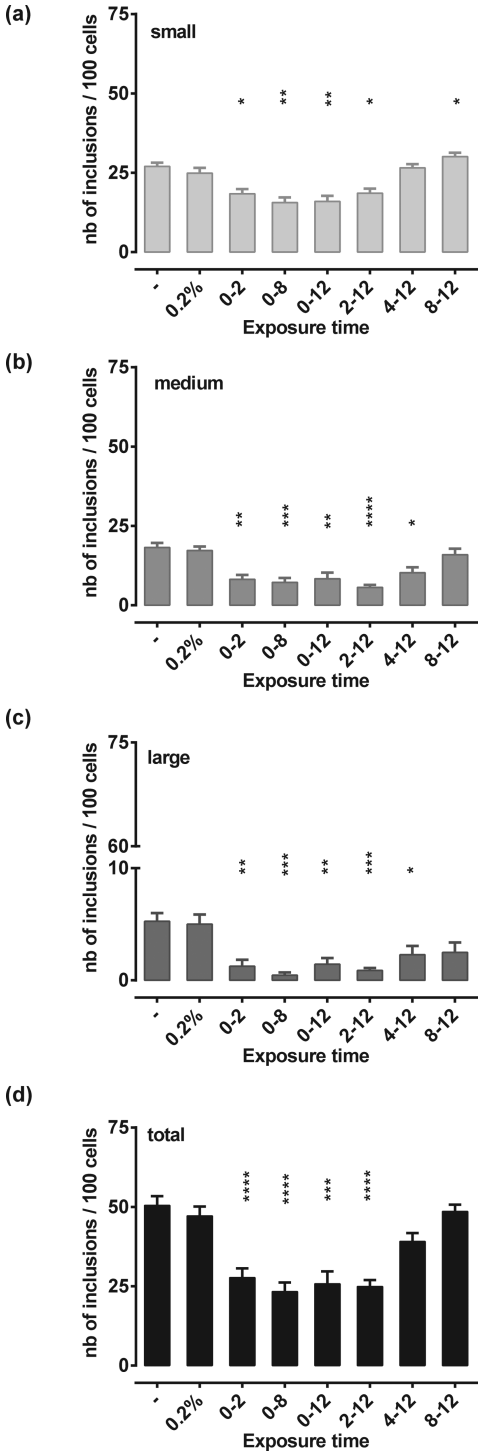

Figure 5. Role of the cytoskeleton in the replication of *E. lausannensis*. (a) The number of small inclusions was reduced upon early or prolonged exposure to 10 μM nocodazole and 20 μM cytochlasin D. (b) The effect is even more pronounced on medium-sized inclusions. (c) For large inclusions, even late treatment with both drugs caused a reduction of the number of inclusions. (d) Total amount of inclusions is significantly reduced in infected cells treated early or throughout the infection with both drugs. Cells were fixed at 12 h p.i. and number of inclusions was quantified. Inclusions were divided in three categories depending on the area quantified (small, medium, large). Images of fixed cells were taken at 400× magnification with tiling. Data are the means +/− SEM of at least three independent experiments performed in quadruplicates. Significance was tested with an unpaired non-parametric Mann-Whitney test compared to 0.2% DMSO-treated cells ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

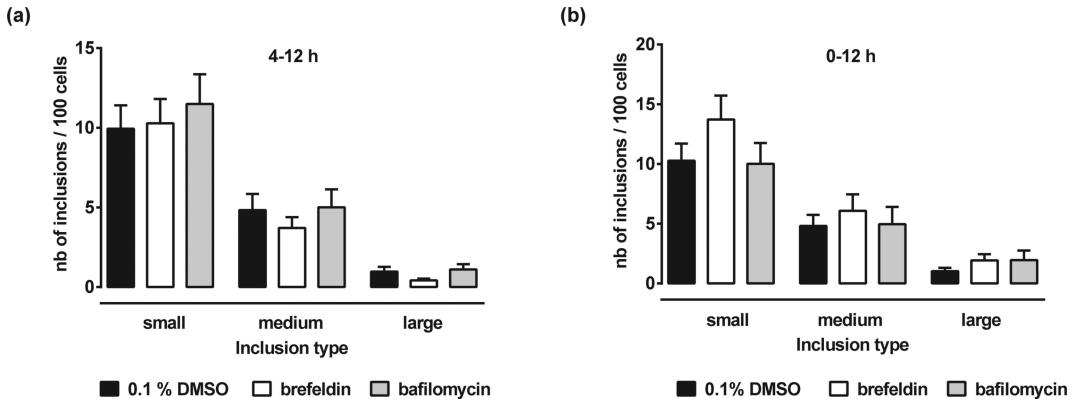

Figure 6. Disruption of ER-Golgi trafficking and lysosomal acidification. **(a)** Infected cells were treated either with the ER-Golgi trafficking disruptor brefeldin A or the lysosomal acidification inhibitor bafilomycin A during 8 h starting 4 h p.i. None of the drugs affected the number of *E. lausannensis* small, medium or large inclusions. **(b)** Treatment with either drug started immediately after internalization and prolonged during 12 hours did not affect the number of small, medium or large inclusions.

E. lausannensis was only reduced and not completely abolished (Fig. 5d).

Lysosomal acidification and ER-Golgi trafficking are not required for *E. lausannensis* replication

Although, most of the *Chlamydiaceae* escape the endosomal route early, the lysosome is an important source of nutrients and lysosomal acidification is crucial for chlamydial replication (Ouellette *et al.* 2011). Since none of the organelles analyzed associated with the BCV, we disrupted lysosomal acidification with bafilomycin A to determine its role as a nutriment source for *E. lausannensis*. The replication of *E. lausannensis* was not affected by the block of lysosomal maturation, independently of the stage of replication (Fig. 6a and b). There was no accumulation of small inclusions, which reinforces the previous results that *E. lausannensis* escapes the lysosomal degradation early.

Even if the disruption of the Golgi observed in our previous experiments could be due to the cytopathic effect of *E. lausannensis*, the ER-Golgi trafficking route could still play an important role in nutrient acquisition, as was observed by Hackstadt, Scidmore and Rockey (1995), for *C. trachomatis*. COPI-dependent trafficking is important in *W. chondrophila* replication, despite an intact Golgi in infected cells (Croxatto and Greub 2010). The ER-Golgi trafficking was disrupted with brefeldin A just after infection, or after maturation into RBs. In both cases, the presence of brefeldin A did not significantly affect *E. lausannensis* replication (Fig. 6a and b).

DISCUSSION

In this work, we showed that *E. lausannensis* replicates in human macrophages and escapes early the endocytic pathway. *Estrella lausannensis* induces a strong cytopathic effect early on during infection preventing the completion of the replication cycle in the majority of the infected cells. Absence of apoptosis inhibition has also been observed with members of the *Parachlamydiaceae* family (Sixt *et al.* 2012). *Parachlamydia acanthamoebae* for example replicates only to a very limited extent within macrophages and causes a rapid cell death (Greub, Mege and Raoult 2003; Greub *et al.* 2005; Roger *et al.* 2010). When apoptosis is blocked with a pan-caspase inhibitor, the bacterial growth is greatly improved.

Disparities in the control of programmed cell death (PCD) might explain the differences in the ability of *Chlamydia*-related bacteria to grow, within macrophages. *Waddlia chondrophila* is rapidly growing within human macrophages without early cytopathic effect (Goy, Croxatto and Greub 2008), whereas *P. acanthamoebae* causes a rapid cytopathic effect limiting its replication (Greub, Mege and Raoult 2003). By comparison, *E. lausannensis* displays an intermediary growth phenotype in human macrophages with rapid growth limited by macrophage PCD. To what extent the lack of PCD control by *E. lausannensis* is involved in growth restriction remains to be determined. A reduced metabolic synthesis is an alternative hypothesis that may explain this reduced growth (Kebbi-Beghdadi, Batista and Greub 2011), supported by genomic data that demonstrate a reduced number of metabolic pathways in the genome of *E. lausannensis* (Bertelli *et al.* 2015) compared to those encoded by the *Waddlia* genome (Bertelli *et al.* 2010).

Estrella lausannensis is leaving the endocytic pathway early upon infection. Escaping the endosomal network early is a strategy developed by many intracellular bacteria to avoid degradation. *Mycobacterium tuberculosis* prevents EEA1 accumulation in the vacuole, therefore preventing further maturation into phagolysosomes (Fratti *et al.* 2001). Only about 10% of the mycobacterial vacuoles are EEA1 positive compared to 30% with latex beads at 10 min p.i. (Fratti *et al.* 2001). These results are very similar to the ones obtained with *E. lausannensis* at 15 min p.i.

Interestingly, *C. trachomatis* trafficking varies depending on the cell line used. In epithelial cells, the bacterial inclusions do not recruit early endosomal markers such as EEA1 and Rab5 (Heinzen *et al.* 1996) and prevent the acquisition of lysosomal and fluid phase markers. However, in macrophages, *C. trachomatis* localizes rapidly in Rab7 positive compartments with increased acidity. Moreover, *C. trachomatis* (serovar L2) has only a very limited replication in macrophages (Sun *et al.* 2012). A similar behavior is observed for *P. acanthamoebae* in macrophages, with LAMP-1 accumulation and Lysotracker staining in the early steps of infection (Greub *et al.* 2005). *Estrella lausannensis* does not recruit early endosomal markers and additionally escapes the fusion with lysosomes, since there is no accumulation of LAMP-1, unlike what is observed for *C. trachomatis* (Sun *et al.* 2012) or *P. acanthamoebae* (Greub *et al.* 2005). We therefore hypothesize that the relatively reduced replication of *E. lausannensis* (as compared to *W. chondrophila*) is not due to a targeting of the bacteria to lysosomal compartments (like *C. trachomatis* or *P. acanthamoebae* in macrophages) but rather, as mentioned above, to a lack of control of PCD.

The interaction of the maturating chlamydial inclusion with several host organelles differs greatly between the different bacterial species, but depends also on the cell type used for infection. Golgi fragmentation has been observed for *Chlamydiaceae* in epithelial cells with recruitment of Golgi fragments to the chlamydial inclusion (Hackstadt, Scidmore and Rockey 1995). However, macrophages infected with *C. trachomatis* (serovar L2) did not display Golgi fragmentation nor interaction of the inclusion with the Golgi (Sun *et al.* 2012). For *E. lausannensis*, the Golgi was fragmented, but not recruited to the inclusion. Since Golgi degradation occurs in the early steps of apoptosis (Mukherjee *et al.* 2007), prior to nuclear fragmentation, we cannot exclude that the fragmentation observed is due to the cytopathic effect induced by *E. lausannensis*.

The role of ER in *C. trachomatis* infection of HeLa was recently elucidated (Dumoux *et al.* 2012). Several ER markers accumulate in patches within the inclusion and calreticulin marks the inclusion membrane. Since PDI, the ER marker used in our study does not colocalize with the *C. trachomatis* inclusion, but rather forms patches within the inclusion, more detailed studies with Z-stacks of *E. lausannensis* would be useful to determine if the same phenotype is observed for *E. lausannensis*. In contrast, for *W. chondrophila* the PDI and calnexin staining showed a colocalization with the inclusion (Croxatto and Greub 2010). It should be noted that a recent publication by Dumoux *et al.* (2012) clearly shows that depending on the ER protein used, the interaction with the inclusion varies considerably. Other ER markers, like calreticulin or calnexin could be used to further assess the role of ER in *E. lausannensis* inclusion biogenesis.

The requirement for mitochondria at the inclusion varies greatly between members of the *Chlamydiales* order. Inclusions of *C. psittaci*, but not *C. trachomatis* or *C. pneumoniae* are surrounded by mitochondria (Matsumoto *et al.* 1991). The same observation was made for *W. chondrophila* (Croxatto and Greub 2010). Moreover, *C. caviae* replication was dependent on the presence of the Tim–Tom import complex found in mitochondria (Derre *et al.* 2007). For *E. lausannensis*, mitochondrial recruitment is not as pronounced as for *W. chondrophila*.

To recruit and interact with the host organelles, the bacterial inclusion needs to either promote its own movement within the cytoplasm or act on the cytoskeleton to attract the organelles of interest. For *C. trachomatis*, the vacuole is known to migrate to the MTOC near the nucleus (Grieshaber, Grieshaber and Hackstadt 2003). This movement is mediated by a modified dynein–dynactin complex (Grieshaber, Grieshaber and Hackstadt 2003). Since, *E. lausannensis* replication is not affected by the presence of the microtubule-disrupting drug nocodazole, the reorganization of the cell by *E. lausannensis* must occur through a different mechanism. The sensitivity of *E. lausannensis* to cytochalasin D treatment at early time points reveals the importance of actin in *E. lausannensis* replication. So far, actin has been involved in entry and exit of *C. trachomatis* (Carabeo *et al.* 2002; Chin *et al.* 2012; Jiwani *et al.* 2012, 2013) and in mitochondria recruitment of *W. chondrophila* (Croxatto and Greub 2010). For *E. lausannensis*, the reduced replication following cytochalasin D treatment is not due to a decreased entry, since the actin depolymerization was only induced after internalization. Interestingly, the effect of cytochalasin D was not reversible, since removal of the drug after 2 h did not allow complete recovery of replication. Noteworthy, in contrast to what was observed for *W. chondrophila* (Croxatto and Greub 2010), *E. lausannensis* replication was only decreased and could not be completely abolished, even when cytochalasin D was combined with nocodazole. Either *E. lausannensis* is able to use intermediary filaments to recruit and transport the nutriments required for replication or the bacteria can find everything within the replicative vacuole. Since, the replication of *E. lausannensis* is not very efficient, a suboptimal growth might be possible even without an intact cytoskeleton.

As the nature of the *E. lausannensis* inclusion could not be established using several organelles and endosomal markers, the source of nutriments for bacterial replication was further investigated with additional inhibitors, such as bafilomycin A. *Chlamydia trachomatis* and *C. pneumoniae* do not fuse with lysosomes, but are still dependent on intact lysosomal function for replication (Ouellette *et al.* 2011). In *E. lausannensis*, the presence of the lysosomal H^+ ATPase pump inhibitor did not prevent replication, indicating a separate source of free amino acids. The second candidate source was the ER-Golgi transport, which is disrupted with brefeldin A. However, *E. lausannensis* was able to replicate in macrophages treated with brefeldin A. This is in major contrast to the majority of *Chlamydiaceae* for which this trafficking pathway is required. Even for *W. chondrophila*, that does not disrupt the Golgi, this pathway is required for successful replication (Croxatto and Greub 2010). The source of lipids and amino acids for the *E. lausannensis* inclusion remains to be established and appears to rely on cellular pathways and components that differ substantially from those used by other *Chlamydiales*. In conclusion, the inability of *E. lausannensis* to control PCD in infected macrophages and the limited growth within suggest a lack of adaptation of this strict intracellular bacteria to the macrophage.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSPD online.

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